

Titration and Antibodies – The Basics Flow Cytometry Staining Protocol

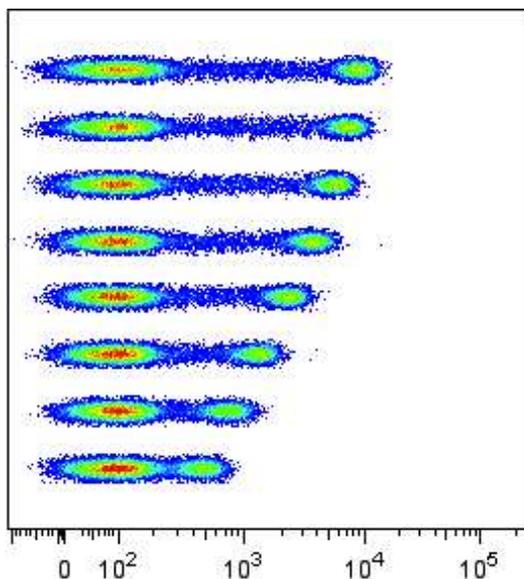
Background: Titration is the process of identifying the correct concentration of antibody to use for a given assay. Also used to ensure the antibody performs within acceptable parameters. Nonspecific antibody binding is best eliminated by optimizing the amount and concentration of the antibody. An antibody titration assay should be applied to determine the antibody amount and concentration resulting in the highest signal of the positive population and the lowest signal of the negative population.

The Why:

The precise amount of an antibody is essential to proper titration.

Too much antibody will result in an increase in the background non-specific (or low affinity) binding, reducing the sensitivity of the fluorescent measurement.

Too little antibody will result in a decrease in the positive signal, again reducing the sensitivity of the fluorescent measurement.



Left: This example shows clearly as the concentration of antibody is added there is greater separation & fluorescent intensity. In the experiment the optimal concentration was 1X (10 μ l)

Left: Leinco Product C119, Anti-Human CD8 (Clone: UCHT-4) - FITC

The How: Titration experiments are easy to perform, and there are several papers that discuss this process (1,2). This protocol has been optimized for 1×10^6 cells in 50 μ l final volume. All staining is performed on ice, in the dark, for 20 minutes.

Identify the 1X concentration. This is Leinco or the manufacturer's recommended concentration for using their antibody in a given assay. You may need to do some calculations to figure out the 1X concentration for this assay. These concentrations can either be a volume (10 μ l per 100 μ l) or a concentration (0.1 μ g/ μ l). Based on the starting concentration, generate a serial dilution series of 4 to 8 points. It is often good to include a 2x concentration to ensure the antibody is working well. If the optimal concentration is 2X the recommended usage, this can suggest a problem.

Solutions Needed:

1. **Staining Buffer:** Phosphate Buffered Saline with 0.5% Bovine Serum Albumin (Fraction V) ([See Leinco Part Number: F1175](#))
2. **Fixation Buffer:** 1-5% formaldehyde in PBS ([See Leinco Part Number: F1194](#))
3. **Blocking Buffer:** 5-10% Normal (species) Serum in Staining Buffer. The species to use is based on the origins of the antibody being used. (Leinco has many Normal Serum species available).

Protocol (using 12x75 mm tubes)

1. Prepare cells at a final concentration of 2.5×10^4 per μl in **blocking buffer**
2. Aliquot 40 μl of cells per sample
 - a. If using a viability dye, include a single stained control for viability (for compensation)
3. Prepare the dilution series
 - a. Prepare the concentration so that 10 μl can be added to each sample
4. Add the antibody to the cells and gently mix.
5. Place on ice, in the dark, for 20 minutes
6. Add 3 ml **staining buffer** to the sample
7. Centrifuge the sample at 900xg
8. Aspirate the solution while not disturbing the pellet
9. Resuspend the sample in $\sim 300 \mu\text{l}$ final volume
 - a. If using viability dye, add to the sample
 - b. If sample has to be fixed, resuspend in $\sim 300 \mu\text{l}$ of **fixation buffer**
 - i. If using fixation buffer, cell impermeant viability dyes (PI, 7AAD, DAPI, etc) cannot be used
10. Analyze the cells on the flow cytometer
 - a. Collect sufficient events for analysis (10,000 positive events)
11. Export the data and analyze in software of choice
12. Calculate the Staining Index and plot versus concentration of antibody(3)

Protocols & Methods Series

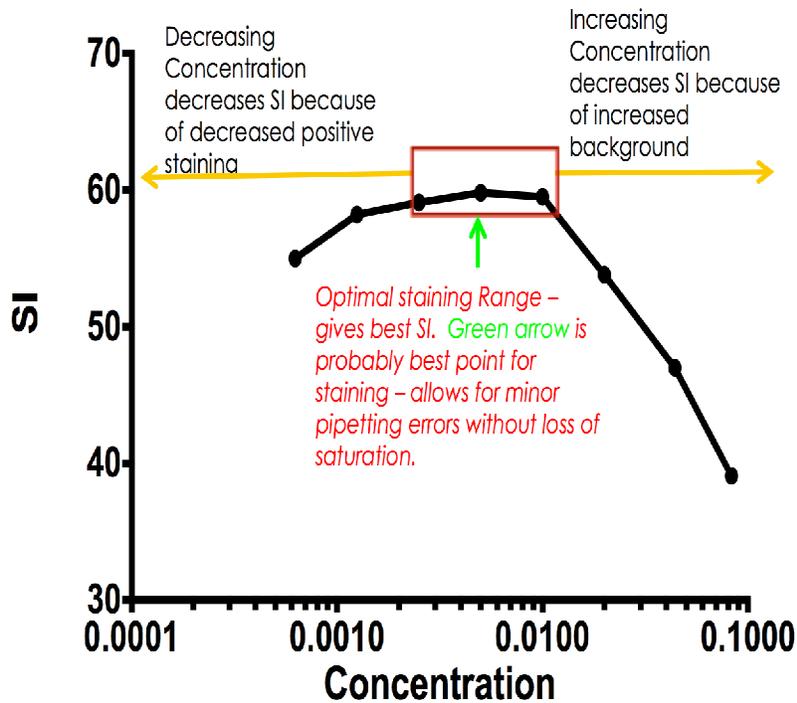
a. Staining Index = $((\text{median}_{\text{pos}} - \text{median}_{\text{neg}}) / ((84\%_{\text{neg}} - \text{median}_{\text{neg}}) * 0.995))$

Protocol (using 96 well plates)

1. Prepare cells at a final concentration of 2.5×10^4 per μl in **blocking buffer**
2. Aliquot 40 μl of cells per sample
 - a. If using a viability dye, make sure to include a single stained control for viability (for compensation)
3. Prepare the dilution series
 - a. Prepare the concentration so that 10 μl can be added to each sample
4. Add the antibody to the cells and gently mix.
5. Place on ice, in the dark, for 20 minutes
6. Add ~300 μl **staining buffer** to each well
7. Centrifuge the plate at 900xg
8. Aspirate (or Flick) the solution out of the wells
9. Repeat steps 6-8 two more times
10. Resuspend the sample in ~200 μl final volume
 - a. If using viability dye, add to the sample
 - b. If sample has to be fixed, resuspend in ~200 μl of **fixation buffer**
 - i. If using fixation buffer, cell impermeant viability dyes (PI, 7AAD, DAPI, etc) cannot be used
11. Analyze the cells on the flow cytometer
 - a. Collect sufficient events for analysis (10,000 positive events)
12. Export the data and analyze in software of choice
13. Calculate the Staining Index and plot versus concentration of antibody(3)
 - a. Staining Index = $((\text{median}_{\text{pos}} - \text{median}_{\text{neg}}) / ((84\%_{\text{neg}} - \text{median}_{\text{neg}}) * 0.995))$

Typical Results

Typical results are shown below.



As is shown in this curve, the titration reveals the optimal concentration (shown in green). As the antibody is increased, the staining index decreases because of the increase in background binding. On the other side, as the concentration decreases, the staining index decreases because the antibody is not at saturating concentrations.

References

1. Hulspas R, O'Gorman MR, Wood BL, Gratama JW, Sutherland DR. Considerations for the control of background fluorescence in clinical flow cytometry. *Cytometry B Clin Cytom* 2009;76:355-64.
2. Maecker HT, Trotter J. Flow cytometry controls, instrument setup, and the determination of positivity. *Cytometry A* 2006;69:1037-42.
3. Telford WG, Babin SA, Khorev SV, Rowe SH. Green fiber lasers: an alternative to traditional DPSS green lasers for flow cytometry. *Cytometry A* 2009;75:1031-9.